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RELATIONSHIPS AMONG SOME PINES FROM SUBGENERA *Pinus* AND *Strobus* REVEALED BY NUCLEAR EST-MICROSATELLITES

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Genetic relationships among 12 taxa from subgenera *Pinus* and *Strobus* were studied through fourteen microsatellite markers, previously developed for *Pinus taeda*. To our knowledge, this is the first comparative study of pines using nuclear EST-microsatellites (EST-SSRs). The total number of detected alleles in all investigated taxa was 72 (5.14 in average). The numbers of alleles per locus and PIC values for estimated markers ranged from 3 to 7, and from 0.43 to 0.81, respectively. Presented results are in accordance with majority of previous genetic investigations and infrageneric classification of genus *Pinus* up to the sectional level, while subsectional position of some species has still not dismissed, especially regarding relict ones. According to nuclear EST-SSRs, *Pinus heldreichii* is in early-diverging position within subsection *Pinaster* and shows the greatest closeness with *P. halepensis*, while *Pinus peuce* doesn't have basal position within subsection *Strobus* being more close to *P. strobus* than to *P. wallichiana.* Furthermore, the closest connections in subsection *Pinus* were found between two *Pinus nigra* subspecies (*dalmatica* and *nigra*) as well as between *P. sylvestris* and *P. mugo.*

Key words: Bosnian pine, Macedonian pine, Austrian pine, nuclear EST-SSR markers

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INTRODUCTION

In investigation of genetic variability and diversity of pines from genus *Pinus* cytogenetic studies (BOGUNIĆ *et al.*, 2011), different protein (AGUNDEZ *et al.*, 1997), and molecular markers (RAPDs, NELSON *et al.*, 1993; RFLPs, DEVEY *et al.*, 1994; AFLPs, TRAVIS *et al.*, 1998, as well as DNA sequences, WANG and WANG, 2014), and their combinations (BOSCHERINI *et al.*, 1994), etc., were used. Microsatellites (ŠARAC *et al.*, 2015; cpSSRs, GÓMEZ *et al.*, 2005), minisatellites (GODBOUT *et al.*, 2010), and their combinations with other molecular (DEVEY *et al.*, 1996; NAYDENOV *et al.*, 2016), or chemical investigations (NAYDENOV *et al.*, 2005), etc., were also reported.

Relationships among pines were revealed previously by vegetative anatomy and plant systematics and cytogenetic (LITTLE and CRITCHFIELD, 1969, and refs. cited therein; SAYLOR, 1964). During time, statements about taxonomy and phylogenetics of pine species were changed according to cytogenetic (SAYLOR, 1972, 1983), biochemical (HERBIN and SHARMA, 1969), and molecular markers: allozymes (KARALAMANGALA and NICKRENT, 1989), isozymes (BERGMANN and GILLET, 1997), RFLPs (STRAUSS and DOERKSEN, 1990), RAPDs (NKONGOLO *et al.*, 2002), nuclear ribosomal DNA (nrDNA) (LISTON *et al*., 1999), chloroplast DNA (cpDNA) (WANG and SZMIDT, 1993; KRUPKIN *et al.*, 1996; WANG *et al*., 1999; PALME *et al.*, 2009) and mitochondrial DNA (mtDNA) gene sequences (WANG and WANG, 2014, etc.), or combinations of markers (GERNANDT *et al.*, 2001; ABRAMOVA, 2002, etc.). Results are approximate but only up to the level of sections, primarily because of the debatable positions and relations of some pines, especially relict ones (*Pinus heldreichii*, *P. peuce* and *P. nigra*).

Pinus heldreichii, Bosnian pine, (Syn. *Pinus leucodermis*), Mediterranean tertiary relict and Balkan subendemite of subgenus *Pinus*, was previously classified into sections: *Diploxylon* (group *Lariciones*) (classification of SHAW, 1914, after LITTLE and CRITCHFIELD, 1969), *Eupitys*, *Pinus* (subsect. *Sylvestres*), *Sula* (subsect. *Canariensis*), etc. (classif. of PILGER, 1926, LITTLE and CRITCHFIELD, 1969, VAN DER BURGH, 1973, respectively, after PRICE *et al.*, 1998). Later, it was placed in subsection *Sylvestre*s (KLAUS, 1989), subsection *Pinus*, or as a sister species to subsection *Pinaster* (PRICE *et al.*, 1998) and, owing to recent genetic research (GERNANDT *et al.*, 2005, 2008, etc.), in subsection *Pinaster* (FARJON, 2017).

Pinus peuce, Macedonian pine, tertiary relict and Balkan endemite of subgenus *Strobus*, previously belonged to subsection *Eustrobi*, group *Strobi*, sections *Strobus* or *Stroboides*, etc. (classif. of ENGELMANN, 1880, SHAW, 1914, 1924, PILGER, 1926, GAUSSEN, 1960, resp., after CRITCHFIELD, 1986). Later biochemical, genetic and phylogenetic studies classified *P. peuce* in sections *Strobus* (LITTLE and CRITCHFIELD, 1969; PRICE *et al.*, 1998; ECKERT and HALL, 2006), *Quinquefoliae* (GERNANDT *et al.*, 2005; 2008; WANG and WANG, 2014) or *Quinquefolius* (FARJON, 2017) and subsections *Strobi*, *Strobus* or *Cembrae* + *Strobi* (GROTKOPP *et al.*, 2004).

Pinus nigra, Austrian or black pine, tertiary relict, was previously classified in subsection *Pinaster* (group *Lariciones*), section *Eupitys* and section *Pinus* (subsection *Sylvestres*) (classifications of SHAW, 1914, 1924; PILGER, 1926; and VAN DER BURGH, resp., after PRICE *et al.*, 1998). *P. nigra* belonged to section *Pinus*, subsection *Sylvestres* (LITTLE and CRITCHFIELD, 1969). Some newer genetic studies (GERNANDT *et al.*, 2005, etc.) put it in subsection *Pinus* (while some pines were transffered in subsection *Pinaster*), which is maintained to this day (FARJON, 2017), but it's relations within subsection are sometimes contradictory (GEORGOLOPOULOS *et al.*, 2016, etc.).

Microsatellites or simple sequence repeats (SSRs) found in all prokaryotic and eukaryotic genomes studied to date are tandemly repeated sequence blocks containing 1-6 DNA bases (YU *et al.*, 2017). Based on their location in the genome, microsatellites can be classified as nuclear (nuSSRs), mitochondrial (mtSSRs) or chloroplastic (cpSSRs) (KALIA *et al*., 2011). SSRs are abundant constituents of non-coding DNA but a large portion of them is located in transcribed regions of the genomes, including protein coding genes and expressed sequence tags (ESTs) (KALIA *et al*., 2011). Microsatellites function in gene regulation, recombination and evolvability (ECHT *et al*., 2011). The use of SSRs as informative genetic markers covers different fields of research like genetic diversity, linkage/association mapping of gene/QTL, marker-assisted selection, variety identification and evolution analysis (JARNE and LAGODA, 1996; YU *et al.*, 2017). Hypervariability, multiallelic nature, codominant inheritance, reproducibility, relative abundance, extensive genome coverage including organellar genomes, chromosome specific location and amenability to automation and high throughput genotyping make microsatellites suitable for many genetic studies (KALIA *et al*., 2011). The major drawback of microsatellites is that they need to be isolated *de novo* from species that have not been previously examined. Such a disadvantage can be made less relevant by choosing the most proper among large number of available methods of microsatellite isolation (ZANE *et al*., 2001). However, there is also an option to circumvent novel microsatellite isolation using their transferability among related species (BARBARÁ *et al*., 2007). Development of microsatellite markers through cross-species amplification of primer sets previously developed for closely-related species is broadly accepted as an alternative approach to *de novo* marker development (e.g. MADUNA *et al*., 2014).

Cross-species amplification of orthologous SSRs among conifers has been suggested to be a valued methodology and applied by several authors (reviewed by GONZÁLEZ-MARTÍNEZ *et al*., 2004). The success of this approach was variable and dependent on numerous issues including the divergence time among species for which genomic SSRs were developed (source species) and species in which SSRs was tested (target species) (GONZÁLEZ-MARTÍNEZ *et al*., 2004).

On the other hand, the availability of genomic resources and EST (expressed sequence tags) databases in conifers enabled development of EST-SSRs found within transcribed but untranslated portion of the genome (e.g. RUNGIS *et al*., 2004; BÉRUBÉ *et al*., 2007). Although EST-SSRs are belived to be less variable than genomic SSRs, they have less null alleles and, as a major advantage, high level of transferability to related species (ELLEGREN, 2004; VARSHNEY *et al*., 2005; HAYDEN *et al*., 2008). To date, pine EST-SSRs have been developed for *P. taeda* (LIEWLAKSANEEYANAWIN *et al*., 2004; BÉRUBÉ *et al*., 2007; ECHT *et al*., 2011), *P. taeda* and *P. pinaster* (CHAGNÉ *et al*., 2004), and *P. contorta* (LESSER *et al.*, 2012).

Relationships among pines obtained by chloroplast SSR markers were previously revealed only by comparing three Mediterranean (BUCCI *et al.*, 1998) and six Iberian pines (SOTO *et al.*, 2010). But, to our knowledge, nuclear EST-microsatellites have never been used in investigations of genetic relationships within genus *Pinus*.

The aim of this study is to establish, on the basis of evaluation of cross-species transfer potential of *P. taeda* EST-SSRs, genetic relationships among pines of subsections *Pinus*, *Pinaster*, *Ponderosae* and *Strobus* (ca. 12 *Pinus* taxa), with special emphasis on three relict species: *Pinus heldreichii*, *P. peuce* and *P. nigra*.

MATERIALS AND METHODS

Plant material

For SSRs analysis, young leaves of 12 taxa of two-needle (G1-G8), three-needle (G9) and five-needle pines (G10-G12) from Serbia, Botanical Garden 'Jevremovac', Belgrade (BGJ), some Belgrade parks (BGP), as well as from Croatia, island Korčula (KOR) were collected (Table 1) and stored frozen (-20^{o} C) until used for DNA isolation.

Table 1. Signes, areals and sources of analysed Pinus taxa.

Sign	*Pinus* taxa	Areal	Collection*
G1	*Pinus mugo*	Southern and Middle Europe	BGP
G2	*Pinus nigra* ssp. *nigra*	Southern Europe	BGP
G3	*Pinus nigra* ssp. *dalmatica*	Dalmatia (Croatia, Europe)	KOR
G4	*Pinus sylvestris*	Eurasia	BGP
G5	*Pinus heldreichii*	Balkans and Italy	BGP
G6	*Pinus halepensis*	Mediterranean region	KOR
G7	*Pinus pinaster*	Mediterranean region	KOR
G8	*Pinus pinea*	Mediterranean region	KOR
G9	*Pinus ponderosa*	Western USA and Canada	BGP
G10	*Pinus peuce*	Balkans	BGJ
G11	*Pinus strobus*	North America	BGJ
G12	*Pinus wallichiana*	South Asia	BGJ

*Collection localities: Belgrade Parks (BGP), island Korčula (KOR), Botanical Garden Jevremovac (BGJ).

Selection of EST-SSRs

EST-SSRs for cross-species PCR amplification in 12 pine taxa were chosen among 53 EST-SSRs reported by CHAGNÉ *et al.* (2004) and 21 ones reported by ECHT *et al.* (2011). The selection criteria were amplification success (CHAGNÉ *et al.* 2004) and number (medium to high) of visible alleles (ECHT *et al.*, 2011). Therefore, we used 12 EST-SSRs of CHAGNÉ *et al.* (2004) developed for *P. taeda* and validated in *P. pinaster*, *P. radiata*, *P. sylvestris*, *P. halepensis*, *P. pinea*, *P. canariensis* (SsPp_cn524, SsrPt_BF778306, SsrPt_ctg1525, SsrPt_ctg3021, SsrPt_ctg4363, SsrPt_ctg5167, SsrPt_ctg7444, SsrPt_ctg7731, SsrPt_ctg8064, RPtest1, RPtest5, RPtest11) and four EST-SSRs of ECHT *et al.* (2011) developed for P. *taeda* (PtSIFG_5015, PtSIFG_5020, PtSIFG_6044 and PtSIFG_6065).

Isolation of DNA, PCR amplifications and electrophoresis

Total genomic DNA was extracted and purified from 0.1 g of leafs by a CTAB method (BASHALKHANOV and RAJORA, 2008). The DNA was quantified and assessed for purity spectrophotometrically, and diluted to a working concentration of 50 ng/µl.

To amplify microsatellites reported by CHAGNÉ *et al.* (2004), PCR reaction mixtures contained 1× Dream Taq Green reaction buffer (Thermo Scientific), 2 mM $MgCl_2$, 0.5 µM of each primer, 0.2 mM of dNTP (Fermentas), 1U of Dream Taq DNA polymerase (Thermo Scientific) and 50 ng of genomic DNA in a total reaction volume of 25 ul. A Biometra Thermocycler TProffesional Standard 96 was set as follows: preliminary denaturing (95°C, 5 min) followed by 30 cycles of denaturing (94°C, 30 s), annealing (locus-specific temperature, 30 s), and extension (72°C, 1 min), as well as a final extension (72°C, 10 min). An additional

touchdown was performed for some loci (10 cycles with the annealing temperature decreasing by 1°C for every cycle).

For the four microsatellite markers, chosen from ECHT *et al.* (2011), the same reaction mixture with the PCR thermocycling protocol used by ECHT *et al.* (2011) on a Biometra Thermocycler TProffesional Standard 96 was employed as follows: 94°C (2 min); 20 cycles of 94°C (30 s), 65°C minus 0.5°C per cycle (30 s), 72°C (1 min); 25 cycles of 2 92°C (30 s), 55°C (30 s), 72°C (1 min 30 s); and 72°C (15 min). Reactions were kept at 4°C until analyzed.

The amplified DNA fragments were separated using 8% polyacrylamide gel electrophoresis for 1.5 hours at 80 mA using Bio-Rad Mini Protean electrophoresis unit. After staining with ethidium-bromide for 30 minutes, gels were photographed under UV light using Biometra BioDocAnalyze Live gel documentation system.

Statistical analyses

Number of allels per locus, gene diversity, heterozygosity, PIC value and average values of these parameters were calculated using PowerMarker V3.25 software. This program was also used for calculations allele frequencies and genetic distances (ROGERS, 1972). For visualization of clusters obtained on the base of genetic distance matrices applying UPGMA method, software MEGA 6.06 was used. Matrices of genetic distances were subjected to Principal Component Analysis (PCA) implemented in STATGRAPHICS Centurion XVI.

RESULTS

Sixteen SSR markers developed for *Pinus taeda* were used to determine whether these markers could be useful to determine genetic diversity in our 12 *Pinus* taxa. The replicated fragments were within the expected size range (size in base pairs). Fourteen markers produced clear and reproducible results (Table 2). For marker SsrPt_ctg1525 amplification was completely missing, while for PtSIFG_5020 the products of replication were non-specific. The total number of detected alleles was 72. The number of alleles per locus ranged from 3 to 7 for different species, while the average value was 5.14. The average values for expected heterozygosity (He) and observed heterozygosity (Ho) were 0.66 and 0.28, respectively (Table 2). Values of PIC for studied markers were high (0.43 - 0.81). Most of the used markers had PIC values above 0.6 (71%), indicating high information value of chosen markers for determining the genetic diversity.

The matrices of genetic distances were used to construct the dendrogram (Fig. 1). The genotypes were sorted into two main clusters which, according to GERNANDT *et al.* (2005), represented two subgenera: *Pinus* and *Strobus*. Furthermore, within the subgenus *Pinus*, species of sections *Trifoliae* and *Pinus* were also separated. Finally, within a section *Pinus*, divergence among species of subsections *Pinus* and *Pinaster* was found.

When genetic distances were imported into principle-component analysis (PCA), species were treated as characteristics (columns) and distance levels as elements (rows). In the plane of first axis of PCA (Fig. 2), which accounted for 53.9% of the total variation, pines of section *Pinus* (two-needle pines), with exception of *P. heldreichii*, were separated from those of section *Trifoliae* (three-needle pines, *P. ponderosa*) and section *Strobus* (five-needle pines, *P. peuce, P. strobus* and *P. wallichiana*). But, species from subsection *Pinaster* were dispersed. Grouping of *P. heldreichii* with two pines from subsection *Pinaster* (*P. pinaster* and *P. pinea*) was obtained in the plane of second axis. *P. halepensis* was associated with species of subsection *Pinus*.

Grouping of five needle pines and their closest position with three-needle *P. ponderosa* were confirmed in both Cluster and PCA (Figs 1 and 2). Furthermore, *P. peuce* was closer to *P. strobus* than to *P. wallichiana*.

Table 2. List of SSR markers used in determination of genetic diversity of 12 Pinus taxa and obtained number of alleles per locus (N), expected heterozigosity (He), observed heterozigosity (Ho) and polymorphic information content (PIC).

No.	Marker	N	He	Ho	PIC
1	SsPp_cn524	7	0.83	0.33	0.80
2	SsrPt_BF778306	3	0.54	0.08	0.43
3	SsrPt_ctg3021	3	0.37	0.00	0.34
4	SsrPt_ctg4363	7	0.83	0.92	0.81
5	SsrPt_ctg5167	7	0.75	0.50	0.72
6	SsrPt_ctg7444	4	0.51	0.08	0.47
7	SsrPt_ctg7731	7	0.80	0.25	0.77
8	SsrPt_ctg8064	4	0.61	0.58	0.53
9	RPtest1	3	0.40	0.00	0.36
10	RPtest5	6	0.66	0.18	0.61
11	RPtest11	5	0.73	0.25	0.69
12	PtSIFG 5015	6	0.78	0.33	0.75
13	PtSIFG 6044	4	0.68	0.00	0.62
14	PtSIFG 6065	6	0.81	0.42	0.78
	Average	5.14	0.66	0.28	0.62

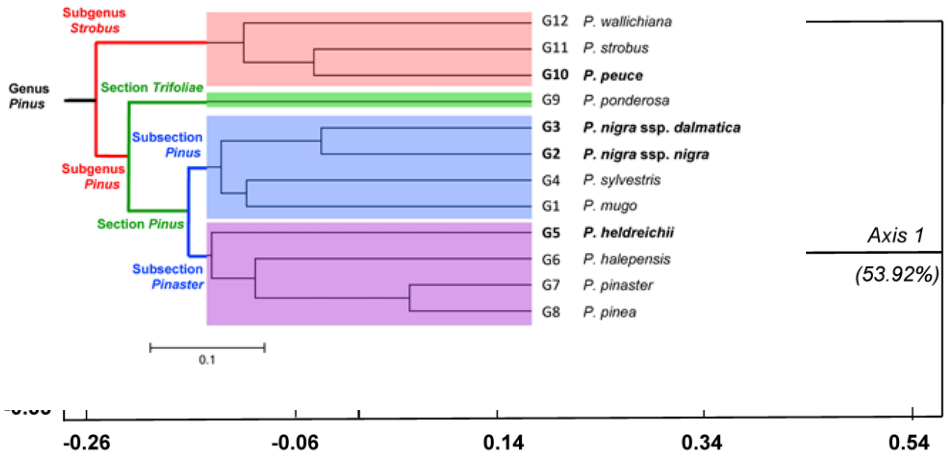

Fig. 1.]

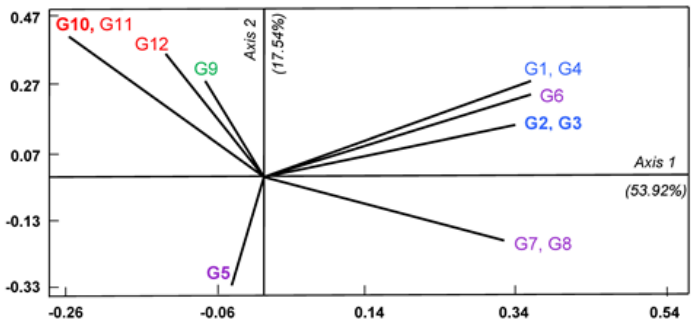

Fig. 2. Principle-component analysis (PCA) of twelve *Pinus* taxa in the plane of first two axes. For details about G1-G12 see Table 1 or Fig. 1.

DISCUSSION

Presented results with EST-SSR markers, which put *P. heldreichii* in early-diverging position within analyzed Mediterannean pines of subsection *Pinaster* (Figs 1 and 2), are in agreement with the majority of genetic (or phylogenetic) studies with protein markers (SCHIRONE *et al.*, 1991), cpDNA (WANG *et al.*, 1999, Fig. 3A; GEADA LÓPEZ *et al.*, 2002; GERNANDT *et al.*, 2003, 2005, 2008; ECKERT and HALL, 2006), nuclear DNA markers (GRIVET *et al.*, 2013), or estimation of genome size (GROTKOPP *et al.*, 2004), but not in studies where new cpDNA fragments were used (trnH-psbA + rbcL, ARMENISE *et al.*, 2012, Fig. 3B; trnV-H/x-h, GEORGOLOPOULOS *et al.*, 2016, Fig. 3C). In our recent study with RAPD markers (KOVAČEVIĆ *et al.*, 2013) *P. heldreichii* clearly diverged from pines of subsection *Pinus* (*P. nigra* and *P. sylvestris*).

However, on the basis of needle morphometry and the content of the methylated flavonols, *P. heldreichii* from Italy (Syn. *P. leucodermis*) was settled in "series" *Sylvestres*, which was considered as a part of subsection *Pinus*, more basal than pines of subsection *Pinaster* (KAUNDUN and LEBRETON, 2010). On the other hand, new and comparative research of terpene composition of several pines of section *Pinus* put *P. heldreichii* in subsection *Pinaster* (MITIĆ *et al.*, 2017).

In presented study *P. heldreichii* is the most similar to *P. halepensis* (Fig. 1) which partially matches only with molecular studies of ARMENISE *et al.* (2012) (Fig. 3B) and ECKERT and HALL (2006), confirming cytogenetic study (SAYLOR, 1964), where *P. heldreichii* was the most similar to *P. halepensis* and *P. brutia*. In other listed investigations closer relationship of *P. heldreichii* with *P. pinaster* and *P. pinea* was found (GRIVET *et al.*, 2013). Comparative terpene studies of MITIĆ *et al.* (2017) suggested closest relation of *P. heldreichii* with *P. pinea.* Our PCA (Fig. 2) also approved the closest connection of *P. heldreichii* with *P. pinaster* and *P. pinea*. But, divergence of *P. heldreichii* from all other investigated pines (Fig. 2) in the plain of first axes could point to it's ancestral position in section *Pinus*, as it was found in some fossil callibrations (GERNANDT *et al.*, 2008).

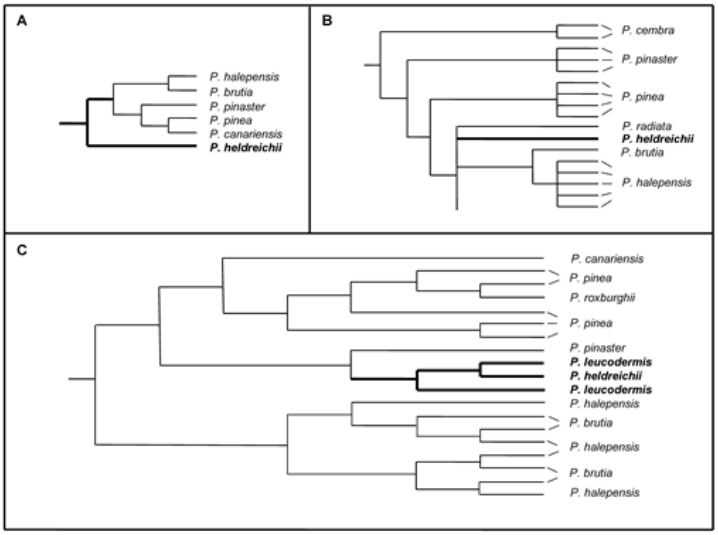

Fig. 3. Relationships among *Pinus heldreichii* and its relatives within subsection *Pinaster* on the basis of molecular markers, after: A) WANG *et al.* (1999); B) ARMENISE *et al.* (2012); C) GEORGOLOPOULOS *et al.* (2016).

In presented results with EST-SSR markers *Pinus peuce*, North American *P. strobus* and Asian *P. wallichiana* were grouped together in subgenus *Strobus* (Fig. 1), section *Quinquefoliae*, subsection *Strobus* (GERNANDT *et al.*, 2005). *Pinus peuce* is more similar to *P. strobus* than to *P. wallichiana*, which has already been found by investigations of isozymes (BELOKON' *et al.*, 1998 after ABRAMOVA, 2002), cpDNA (WANG *et al.*, 1999, Fig. 4B; GERNANDT *et al.*, 2003, 2005; SCOTT, 2004; ECKERT and HALL, 2006), mtDNA (WANG and WANG, 2014; Fig. 4C), nrDNA markers (LISTON *et al.*, 1999), and their combinations (TSUTSUI *et al.*, 2009), or even in combination of molecular and nonmolecular data (GERNANDT *et al.*, 2008). In the most of these and other molecular findings (Fig. 4A; Fig. 4B, and GERNANDT *et al.*, 2008), *P. peuce* was basal species in subsection *Strobus* but not in some recent studies (mtDNA, WANG and WANG, 2014, Fig. 4C). According to our previous results using RAPD markers, *P. peuce* also had early-diverging position, even among all investigated pines of subgenera *Strobus* and *Pinus* (KOVAČEVIĆ *et al.*, 2013). Cytogenetic research of SAYLOR (1983) also pointed to separation of *P. peuce* from other five-needle pines.

Similarity among *P. peuce* and *P. strobus*, obtained in presented study, was not found in some other investigations, when *P. peuce* was at equal large distance from both *P. strobus* and *P. wallichiana* (allozymes, SHURKAL *et al.*, 1992, Fig. 4A); genome size estimation, GROTKOPP *et al.*, 2004; RAPDs, KOVAČEVIĆ *et al.*, 2013), very far from *P. strobus* (cpDNA, PARKS *et al.*, 2009), or even closer to *P. wallichiana* (which was also found more ancestral than *P. peuce*) (cpDNA, WANG and WANG, 2014). Furthermore, some biochemical data also didn't match, such as analyses of phenols, which indicated the similarity among *P. peuce* and North American *P.*

lambertiana, and analyses of xylem resin, which, according to presence of diterpene cembrene, indicated similarity of *P. peuce* with North American *P. albicaulis* and some Asian pines (ERDTMANN, 1963 and MIROV, 1967, respectively, after CRITCHFIELD, 1986).

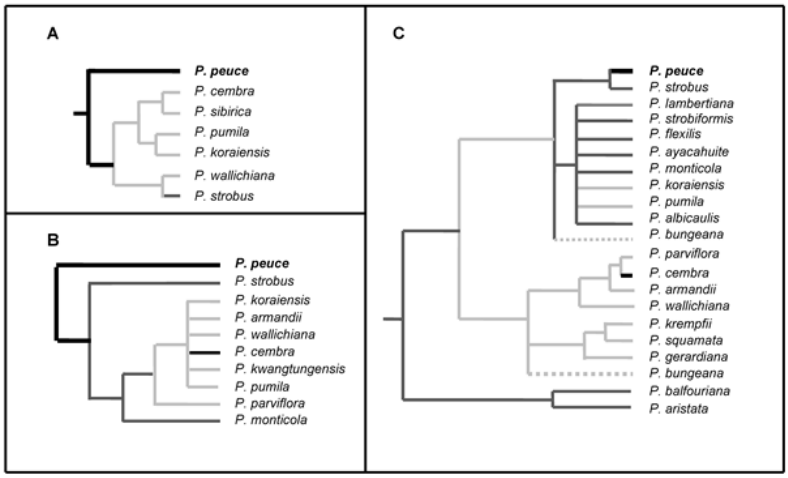

Fig. 4. Relationships among *Pinus peuce* and its relatives within subgenus *Strobus* on the basis of genetic markers, after: A) SHURKAL *et al.* (1992); B) WANG *et al.* (1999); C) WANG and WANG (2014). Origin of *Pinus* species: Europe, ▬ ; America: ▬; Asia: ▬ .

Presented relationships among pines of subsection *Pinus* show the closest connection among two subspecies of *Pinus nigra* (*dalmatica* and *nigra*) with on one, and *P. sylvestris* and *P. mugo*, on the other side (Fig. 1). Grouping of typical *P. nigra* and it's oriental subspecies (*pallasiana*) was also obtained in comparative study of several pines using allozymes (SHURKAL *et al.*, 1992) (Fig. 5A). Furtheremore, similar results were obtained in comparative investigations of larger number *Pinus* species based on terpene markers (MITIĆ *et al.*, 2017), as well as needle morphometry and flavonols (KAUNDUN and LEBRETON, 2010). However, composition of the essential oils and flavonols in occidental/meridional *P. nigra* subspecies: *salzmannii* and *laricio*, was considerably different (MITIĆ *et al.*, 2017; KAUNDUN and LEBRETON, 2010).

In several studies that are included only *P. nigr*a taxa, differences between subsp. *dalmatica* and subsp. *nigra* were found by morphology and anatomy of needles (LIBER *et al*., 2002), flow cytometry (BOGUNIĆ *et al.*, 2003) and RAPD markers (LIBER *et al.*, 2003), but not according to genome size (BOGUNIĆ *et al.*, 2007) and karyotype differentation (BOGUNIĆ *et al.*, 2011). Moreover, if we accept the recent concept (based on cpDNA markers), proposed by NAYDENOV *et al*. (2016), who identified three differentiated genetic formations consisted with European Black Pine's natural distribution (Westerns Mediterranean, the Balkan Peninsula and Asia Minor), ssp. *dalmatica* and ssp. *nigra* belong to same genetic group.

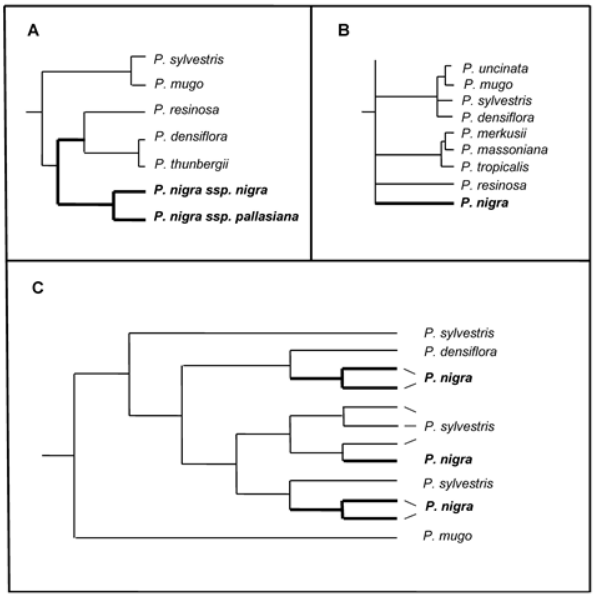

Fig. 5. Relationships among *Pinus nigra* and its closer relatives within subsection *Pinus* on the basis of genetic markers, after: A) SHURKAL *et al.* (1992); B) GERNANDT *et al.* (2005); C) GEORGOLOPOULOS *et al.* (2016).

Similarity of *P. sylvestris* and *P. mugo* obtained by EST-SSR markers fits with results of genetic investigations of seed storage proteins (SCHIRONE *et al.*, 1991), allozymes (SHURKAL *et al.*, 1992), cpDNA sequences (GERNANDT *et al.*, 2005, Fig. 5B; GEADA LÓPEZ *et al.*, 2002; ECKERT and HALL, 2006; ARMENISE *et al.*, 2012) and genome size estimations (GROTKOPP *et al.*, 2004). In most of them, as well as in studies of WANG *et al.* (1999) and GERNANDT *et al.* (2003, 2008), *P. nigra* early-diversed from *P. sylvestris* and *P. mugo* and was also close with some American and/or Asian pines, or even closer with them than with *P. sylvestris* and *P. mugo*, which are sometimes more divergent (SHURKAL *et al.*, 1992, Fig. 5A). Our analysis with EST-SSRs also shows early-diverging of *P. nigra*. In recently published investigation, where new cpDNA fragment was used (GEORGOLOPOULOS *et al.*, 2016, Fig. 5C), even *P. mugo* was more divergent than *P. nigra* and *P. sylvestris*. It was also found in chronogram made by ECKERT and HALL (2006) and cytogenetic study (SAYLOR, 1964).

CONCLUSIONS

Relationships among 12 taxa of the genus *Pinus* revealed by EST-SSRs are in accordance with the majority of previous genetic investigations and infrageneric classification of genus *Pinus*.

Early-diverging position of *Pinus heldreichii* in subsection *Pinaster* obtained by EST-SSRs, fits with results of most other molecular studies except with newer ones where *P. pinaster* or some other pines were more divergent. Also, obtained greater similarity of *P. heldreichii* with

P. halepensis fits with only a few studies, while in others are more similar to *P. pinaster* or *P. pinea*. Closer relationship of *Pinus peuce* to *P. strobus* than to *P. wallichiana* obtained by EST-SSRs sometimes fit with other molecular studies, but sometimes *P. peuce* was in early-diverging position or, even more, the most divergency of *P. strobus* and/or *P. wallichiana* were found. According to EST-SSRs, *Pinus nigra* has the smallest distance with its subsp. *dalmatica* which fits with some of other molecular studies. Early-diverging of *P. nigra* from *P. sylvestris* and *P. mugo* fits with most previous investigations, but not with newer molecular studies where *P. mugo* was more basal.

The presented data show that use of different genomes and their sequences (molecular markers) is constantly changing the picture on genetic and phylogenetic relationships within the genus *Pinus*, and this is also projected on relationships among our studied species at the subsection level. Even two newer fossil-based calibration studies (ECKERT and HALL, 2006; GERNANDT *et al.* 2008) fit neither with each other nor with most other studies. Diversification of genus *Pinus* to subgenera *Pinus* and *Strobus* took place in early or late Cretaceous.

Our results of EST-SSRs, as well as numerous other studies of genetic relationships among pines, are still not sufficient for understanding of their phylogeny and relationships as a whole. Discordant mtDNA and cpDNA phylogenies of genus *Pinus* (WANG and WANG, 2014) additionally complicated these comphresive researches. Similar problem was found in *Picea* (BOUILLÉ *et al.*, 2011). In this genus even simultaneous study of all three genomes (nuclear, plastid and mitochondrial) was not enough for complete phylogenetic picture of spruces, pointing to further investigation of larger genomic data (LOCKWOOD *et al.*, 2013). So, in order to achieve complete picture of genetic relationships and phylogeny of relict, extant and extinct pines, it is necessary to consider current knowledge about their morphology, taxonomy, biogeography, biochemistry, genetics, phylogeny and evolution and combine them with contemporary genome investigations. At the same time it is also necessary to consider the fact that evolution of species is also followed by evolution of genome (HIRAO *et al*., 2008; RAN *et al.*, 2010), which may lead to additional complications in reaching valid conclusions on relations within investigated taxa, in this case the genus *Pinus*.

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ODNOSI IZMEĐU NEKIH BOROVA PODRODOVA *Pinus* I *Strobus* UTVRĐENI POMOĆU JEDARNIH EST-MIKROSATELITA

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Izvod

Proučavane su genetičke veze između 12 taksona podrodova *Pinus* i *Strobus* putem 14 mikrosatelitnih markera, prethodno razvijenih kod *Pinus taeda*. Prema našim saznanjima, to je prvo uporedno proučavanje borova putem jedarnih EST-mikrosatelita (EST-SSRs). Ukupan broj detektovanih alela za sve vrste je bio 72 (u proseku 5.14). Broj alela po lokusu i PIC vrednosti bile su 3-7 i 0.43-0.81, respektivno. Prezentovani rezultati se uklapaju sa većinom prethodnih genetičkih istraživanja i infrageneričkom klasifikacijom roda *Pinus* do nivoa sekcija, dok položaj vrsta u subsekcijama još uvek nije razrešen, naročito u pogledu reliktnih vrsta. Prema jedarnim EST-SSR markerima, *Pinus heldreichii* je u rano-suprostavljenoj poziciji unutar podsekcije *Pinaster* i pokazuje najveću bliskost sa *P. halepensis*, dok *Pinus peuce* nema bazalni položaj unutar podsekcije *Strobus* i bliži je *P. strobus* nego *P. wallichiana*. Osim toga, najbliže veze u podsekciji *Pinus* su se pokazale između dve podvrste *Pinus nigra* (*dalmatica* i *nigra*) kao i između *P. sylvestris* i *P. mugo*.

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